

Remarks

Upon entry of the present amendment, claims 24-42 will be pending. Claims 9, 11, and 13-23 have been canceled without prejudice or disclaimer. Applicants reserve the right to pursue the canceled subject matter in a later filed continuing application.

New claims 24-42 have been added to more clearly define the subject of the instant invention. Support for new claims 24-42 can be found throughout the specification as filed. In particular, support for the new claims can be found in the specification, for example at page 18, paragraph 56; at page 18, paragraph 60; at page 24, paragraph 79; at page 21, paragraph 69; at page 23, paragraph 77; and at original claim 9. Accordingly, no new matter has been added.

Applicants thank the Examiner for the telephone interview with Applicants' representative, Melissa Pytel, on May 3, 2005. Amendments and remarks made herein are in accordance with the suggestions discussed during the telephone interview.

I. Enablement Rejections Under 35 U.S.C. § 112, First Paragraph

a. The Examiner has maintained the rejection of claims 9, 13-17, and 19-23 under 35 U.S.C. § 112, first paragraph for alleged lack of enablement in that the claims allegedly fail to comply with the deposit requirement rules. In particular, the Examiner notes that although Applicants stated in the response to the Patent Office mailed on December 20, 2004 (hereinafter "the December 20, 2004 response") that a partially redacted copy of the ATCC Deposit Receipt for ATTC Deposit No. 209038 was enclosed as Exhibit A, Exhibit A did not accompany the response. See, section 4, pages 2-3.

In order to correct this error, Applicants submit herewith a partially redacted copy of the ATCC Deposit Receipt for Accession Number 209038 as Exhibit A that was erroneously omitted from the December 20, 2004 response.

Applicants submit that the rejections under 35 U.S.C. 112, first paragraph, have been overcome or obviated by the amendments and statements made above and in the December 20, 2004 response. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw this rejection.

b. The Examiner has maintained the rejection claims 9, 13-17, and 19-23 under 35 U.S.C. § 112, first paragraph for alleged lack of enablement. In particular, the Examiner

maintains that the specification does teach how to make or use any I-FLICE-2 polypeptide with 95% identity to fragments of SEQ ID NO:6 because the specification allegedly fails to teach which amino acids within the fragments can be "substituted, deleted, added or any combination thereof and whether the resulting polypeptide maintains its anti-apoptotic activity." *See*, section 5, page 4, second paragraph. Moreover, the Examiner alleges that because "the term 'percent' is relative and can be defined by the algorithm and parameter values set when using the algorithm used to compare the sequences" and "because applicants have not disclosed the specific condition used to score sequence identity", making and using polypeptides with 95% identity to fragments of SEQ ID NO:6 would require undue experimentation. *See*, section 5, page 5, first paragraph.

Applicants respectfully disagree and traverse this rejection.

Preliminarily, Applicants thank the Examiner for noting that the references cited in the December 20, 2004 response as Exhibit B could not be found in the papers accompanying the response. Applicants respectfully submit herein the reference, Gayle *et al.*, "Identification of regions in Interleukin-1 α important for activity," *J. Biol. Chem.* 268(29):22105-22111(1993) as Exhibit B in order to correct this obvious error.

In addition, in the telephone interview with Applicants' representative, the Examiner indicated that she would reconsider and withdraw the rejection requiring Applicants to "disclose the specific condition used to score sequence identity" (*id*) if Applicants canceled claims reciting percent identity to fragments of SEQ ID NO:6. Although Applicants maintain that a rejection requiring Applicants to provide the algorithm for determining percent identity is improper, in the interest of facilitating prosecution, and in no way in acquiescence to the Examiner's rejection, Applicants provide herewith an amended claim that no longer encompasses percent identity to a fragment of SEQ ID NO:6. Applicants reserve the right to pursue the canceled subject matter in a later filed continuing application. Accordingly, Applicants contend that the Examiner's rejection has been overcome and respectfully request the Examiner to reconsider and withdraw this rejection.

Moreover, Applicants contend the specification enables one of skill in the art to make and use the subject matter of the currently pending claims. Applicants reiterate that the Federal Circuit has held that making the claimed species and screening them for function is acceptable, as long as the experimentation is not undue. As in all cases, this is the test: whether it would require undue experimentation to practice the invention – even when a

claim might encompass some inoperative embodiments. *See generally, Atlas Powder v. E.I. Du Pont de Nemours & Co.* 750 F.2d 1569, 224 U.S.P.Q. (BNA) 409 (Fed. Cir. 1984).

Therefore, it is clearly not *per se* undue to make polypeptides that are 95% identical to either the amino acid sequence of SEQ ID NO:6 or the amino acid sequence encoded by the cDNA contained in ATCC Deposit No. 209038 and then test these variants for the ability to inhibit TNFR-1 and CD-95 induced apoptosis, particularly when specific guidance was clearly disclosed in the specification, coupled with what was known in the art at the time the invention was filed. Moreover, such guidance need not be provided through working examples in the specification. *See, M.P.E.P. § 2164.02 at 2100-187.*

Applicants reiterate that ample guidance is provided in the specification on how to make, test, and use the claimed polypeptides to inhibit TNFR-1 and CD-95 induced apoptosis. For example, the specification teaches that I-FICE-2 polypeptides of the invention and variants thereof are capable of inhibiting TNFR-1 and CD-95 induced apoptosis. *See, specification, for example, at page 3, paragraph 5; at page 4, paragraph 9; at page 7-8, paragraph 23; and at page 18, paragraph 59.* The specification further teaches how to make, screen, and use the claimed polypeptides.

In addition to the amino acid sequence common to the polypeptides of the claimed invention (e.g., SEQ ID NO:6), the specification further provides ample disclosure of other relevant characteristics of the claimed polypeptides. First, the specification provides the parameters used to determine the percent identity of the polypeptides of the claimed invention. *See, for example, specification at page 21, paragraph 67 to page 22, paragraph 71.* The specification further provides a detailed analysis of the structural attributes of the I-FLICE-2 protein, including important domains of the protein. *See, for example, specification at page 7, paragraph 23.* Moreover, the specification provides guidance on how to make variants which fall within the scope of the instant invention by insertion, deletion, inversions, repeats, and type substitutions. *See, specification, for example, at page 18, paragraph 59 to page 22, paragraph 71.* The specification also provides a detailed analysis of the functional attributes of the I-FLICE-2 protein, such as, for example, antigenic index of the I-FLICE-2 polypeptide. *See, specification, for example, at page 5, paragraph 17; and at Figure 5.* Thus, the specification provides ample direction to the skilled artisan as to which amino acids of SEQ ID NO:6 are suitable to modify without substantially affecting activity.

Additionally, the specification also teaches the skilled artisan how to screen these variants for the ability to inhibit TNFR-1 and CD-95 induced apoptosis. In particular, the specification teaches a cell death assay useful for measuring the apoptotic activity of I-FLICE-2 polypeptides. See, specification, for example, at page 5, paragraph 18; at page 15, paragraph 46, at Example 6, page 54, paragraph 192 to page 55, paragraph 194.

Accordingly, Applicants submit that the teachings of the specification, in combination with the level of skill in the art, enable a skilled artisan to make by substitution, deletion and insertion, polypeptides that are 95% identical to either the amino acid sequence of SEQ ID NO:6 or the amino acid sequence encoded by the cDNA contained in ATCC Deposit No. 209038 without changing the anti-apoptotic activity of the protein; to screen said polypeptides for anti-apoptotic activity; and to use said polypeptides in inhibiting TNFR-1 and CD-95 induced apoptosis. Thus, Applicants submit that one of skill in the art would be able to routinely make and use the invention commensurate in scope with the claims. Any experimentation, if necessary, would not be undue. Accordingly, Applicants respectfully request that the Examiner's rejection of the claims 9, 13-17, and 19-23 under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

Conclusion

Applicants respectfully request that the above-made remarks be entered and made of record in the file history of the instant application. The Examiner is invited to call the undersigned at the phone number provided below if any further action by Applicant would expedite the examination of this application.

If there are any fees due in connection with the filing of this paper, please charge the fees to our Deposit Account No. 08-3425. If a fee is required for an extension of time under 37 C.F.R. § 1.136, such an extension is requested and the fee should also be charged to our Deposit Account.

Dated: May 5, 2005

Respectfully submitted,

By 

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Exhibit A



American Type Culture Collection

12301 Parklawn Drive • Rockville, MD 20852 USA • Telephone: 301-231-5519 or 231-5532 • FAX: 301-816-4366

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

MAY 15 1997

HGS PATENT DEPT.

To: (Name and Address of Depositor or Attorney)

Human Genome Sciences, Inc.
Attn: Robert H. Benson
9410 Key West Avenue
Rockville, MD 20850

Deposited on Behalf of: Human Genome Sciences, Inc.

Identification Reference by Depositor:

ATCC Designation

DNA Plasmid 1631454 (PF 381)

209038✓

The deposits were accompanied by: a scientific description a proposed taxonomic description indicated above. The deposits were received May 15, 1997 by this International Depository Authority and have been accepted.

AT YOUR REQUEST: ☒ We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested May 22, 1997. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Barbara M. Hailey
Barbara M. Hailey, Administrator, Patent Depository

Date: May 22, 1997

Identification of Regions in Interleukin-1 α Important for Activity*

(Received for publication, April 28, 1993, and in revised form, June 23, 1993)

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Saturation mutagenesis of the mature human interleukin-1 α (IL-1 α) gene has been performed. Following expression in *Escherichia coli*, the biological and receptor binding activities of the mutant proteins were examined. Most of the molecule could be altered with little effect on either function. More than 3,500 mutants were examined, and only 23 unique amino acid sequences were identified which resulted in an altered ratio of biological to binding activity when compared with wild-type IL-1 α . These proteins possessed mutations at 38 of the 159 amino acid residues in IL-1 α . Random mutagenesis at several of these positions identified further substitutions that affected activity. Examination of a model for IL-1 α localized most of the residues which altered activity along one face of the molecule. This region appears to be distinct from areas of IL-1 which have been postulated to make contact with IL-1 receptor.

Interleukin-1 (IL-1)¹ is a potent cytokine that is involved in inflammatory responses and affects the growth and differentiation of T cells, B cells, and fibroblasts (for review, see Durum *et al.*, 1985). The two molecules responsible for this activity, IL-1 α and IL-1 β , share only 22% amino acid similarity (March *et al.*, 1985; Auron *et al.*, 1987). Each binds to both forms of IL-1 receptor (Dower *et al.*, 1986; McMahan *et al.*, 1991). Both IL-1 molecules are produced as intracellular precursors and are subsequently processed to mature proteins. Although the precursor form of IL-1 α is biologically active, only the mature form of IL-1 β has any biological activity (Mosley *et al.*, 1987a, 1987b). cDNAs have been isolated encoding a third form of IL-1, IL-1 receptor antagonist (IL-1ra) (Carter *et al.*, 1990; Eisenberg *et al.*, 1990; Hannum *et al.*, 1990). This molecule has homology to both IL-1 α and IL-1 β and has an affinity for the IL-1 receptors close to that seen for IL-1 α and IL-1 β , yet elicits no biological response from target cells (Arend *et al.*, 1990; Carter *et al.*, 1990; Eisenberg

et al., 1990; McMahan *et al.*, 1991). Alignments among these three sequences for several species do not indicate which residues are important for activity (Yanofsky and Zurawski, 1990). The three-dimensional structures of IL-1 α (Graves *et al.*, 1990) and IL-1 β (Gilliland *et al.*, 1987; Priestle *et al.*, 1990) demonstrate the structural similarity of the two molecules but do not suggest which regions of the molecules are responsible for activity.

Deletion and combinatorial mutagenesis have identified residues at the amino terminus of IL-1 α which are needed for biological activity (Yanofsky and Zurawski, 1990). However, since the affinities of these mutants for IL-1 receptors were not examined, it is impossible to differentiate whether these mutations affect biological activity, the ability to bind IL-1 receptor, or the structural integrity of the protein. The existence of IL-1ra demonstrates the ability to separate biological activity from binding activity. Although many site-directed mutations of IL-1 α and IL-1 β have little effect on the function of the proteins (Gronenborn *et al.*, 1988; Kamogashira *et al.*, 1988a, 1988b; Craig *et al.*, 1989), several mutants demonstrate greatly reduced biological activity with little change in affinity for the type I IL-1 receptor (Gehrke *et al.*, 1990; Yamayoshi *et al.*, 1990).

Using a novel method of saturation mutagenesis, random mutations were generated throughout the entire sequence of IL-1 α . Assays to determine both biological and binding activity were performed on several thousand mutant proteins. By examining the ratio of biological to binding activity for each mutant and comparing it with the ratio for wild-type IL-1 α , regions of IL-1 α which affect these two properties differentially were identified.

MATERIALS AND METHODS

Enzymes and Vectors—All restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were obtained from Boehringer Mannheim or New England Biolabs. BBG1, a plasmid bearing a synthetic gene for human IL-1 α , was purchased from British Biotechnology. The construction of pPLBBGIL-1 α has been described previously (Poindexter *et al.*, 1991). An *SpeI* site was added using site-directed mutagenesis. Plasmid DNA was purified by the alkaline lysis method (Ausubel *et al.*, 1988).

DNA Synthesis—Oligonucleotide cassettes used for the construction of mutants were synthesized on an Applied Biosystems model 380A DNA synthesizer. For saturation mutagenesis each of the four phosphoramidites was contaminated with a small amount of the other three. The phosphoramidites were contaminated at two different levels, 4.2% for the sense strand and 8.4% for the antisense strand (Poindexter *et al.*, 1991). For random mutagenesis, the cassette was synthesized normally except for the substitution of an equimolar mixture of the four phosphoramidites for the three nucleotides making up the chosen codon. Oligonucleotides were purified by polyacrylamide gel electrophoresis on a 40-cm 8% polyacrylamide, 7 M urea gel. Care was taken to excise full-length oligonucleotides, and the

* This work was supported by funding from a joint venture between Immunex Corporation and Eastman Kodak. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X55445.

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¹ The abbreviations used are: IL-1, interleukin-1; IL-1ra, interleukin-1 receptor antagonist.

FIG. 1. Coding sequence of human IL-1 α gene used in saturation mutagenesis. The DNA sequence downstream from the promoter of pPLBBGIL-1 α is shown (accession no. X55445). The coding sequence is represented by capital letters. The coding sequence for the IL-1 α gene is divided into eight regions, named for the restriction enzymes that border them. The nine restriction enzymes are *Cla*I (C), *Eco*RI (R), *Pst*I (P), *Sst*II (S), *Pvu*II (U), *Bam*HI (B), *Spe*I (S), *Bgl*II (G), and *Hind*III (H). Thus the eight regions are CR, RP, PS, SU, UB, BS, SG, and GH.

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          ClaI (C)      M S A P F S F L S N V K Y N F M R
1 atcgatactATGTCAGCACCTTTTAGCTTCCTGAGCAATGTGAATACAACCTTTATGAGG 60
          EcoRI (R)
61 ATCATCAAAATACGAATTCATTCTGAACGATGCATTGAACCACTTATTATTCGTGCAAC 120
          PstI (P)
121 GACCAGTACCTGACTGCAGCAGCCCTGCACAATCTGGACGAAGCAGTTAAATTCGACATG 180
          SstII (S)
181 GGTGCTTACAAGAGCTCGAAAGACGACGCAAAATCACTGTAATCCTACGTATTCTTAA 240
          PvuII (U)
241 ACCCAGCTGTATGTATTAQDEEDQPVLLKEMPT 300
          BamHI (B)
301 GAGATCCCGAAGACTATCACTGGATCCGAGACTAACCTGCTTCTTCTGGGAAACTCAC 360
          SpeI (S)
361 GGTACCAAAACTACTTCACTAGTGTGGCTCATCCGAACCTGTTTCATCGCGACAAAACAG 420
          BglII (G)
421 GACTACTGGGTATGCTGGCAGGCGGTCCGCCATCGATCACTGACTTCCAGATCCTCGAG 480
          HindIII (H)
          N Q A * *
481 AACCAAGCATAATAAgaatctaagctt 506
  
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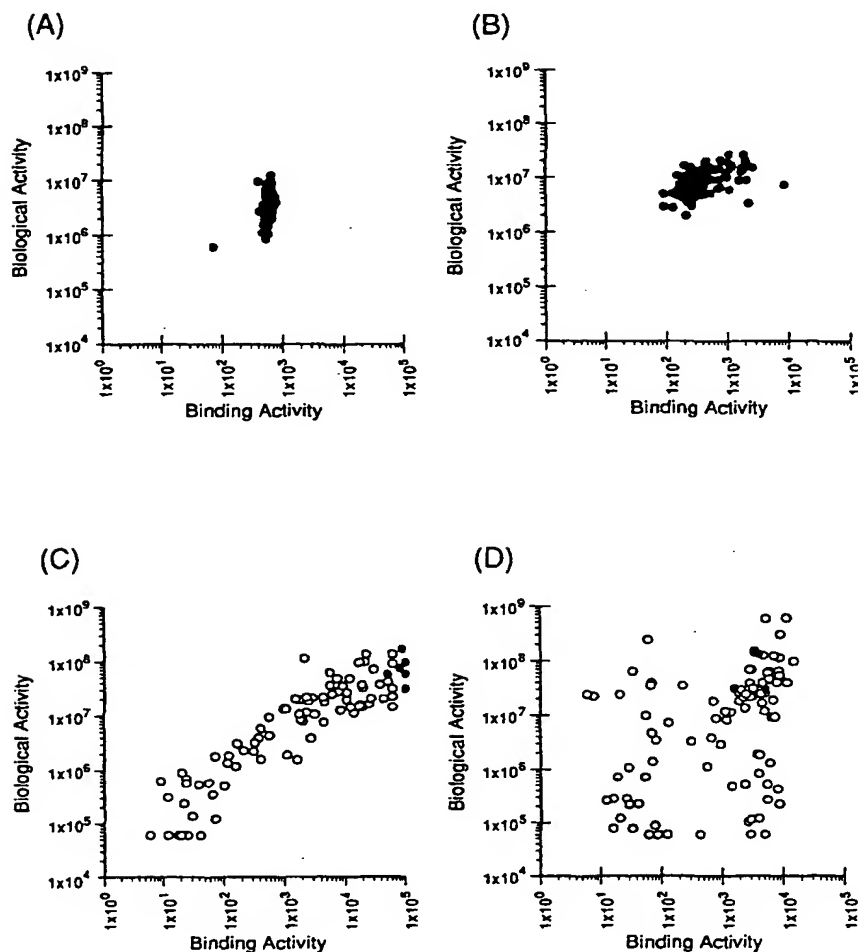


FIG. 2. Ability of biological and binding activity screens to identify mutants with altered activity. Panel A, 96 identical samples of wild-type IL-1 α . Panel B, 96 independent samples of wild-type IL-1 α . Panel C, 90 mutants of IL-1 α from the PS region (open circles) and 6 wild-type IL-1 α (closed circles). Panel D, 90 mutants of IL-1 α from the RP region (open circles) and 6 wild-type IL-1 α (closed circles). The biological activity is expressed in units/ml, and the binding activity is the reciprocal dilution that results in 50% binding inhibition (see "Materials and Methods").

oligonucleotides were deprotected and resuspended in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA).

Assembly and Cloning of Mutagenic Oligonucleotides—The general procedure has been described (Poindexter *et al.*, 1991). Twenty picomoles of each oligonucleotide in a mutagenic cassette were mixed in 20 μ l of TE and placed at 65 $^{\circ}$ C for 15 min. The mixture was allowed to cool slowly to room temperature and then placed on ice. Each

mutagenic cassette had unique ends, allowing them to be ligated into appropriately cleaved vectors. To increase the efficiency of screening, intermediate vectors were constructed for each region. These intermediate vectors contained an irrelevant segment of DNA inserted between the relevant restriction enzyme sites. Insertion of the mutagenic cassette followed by restriction with an enzyme unique to the intermediate plasmid greatly reduced the incidence of vectors without

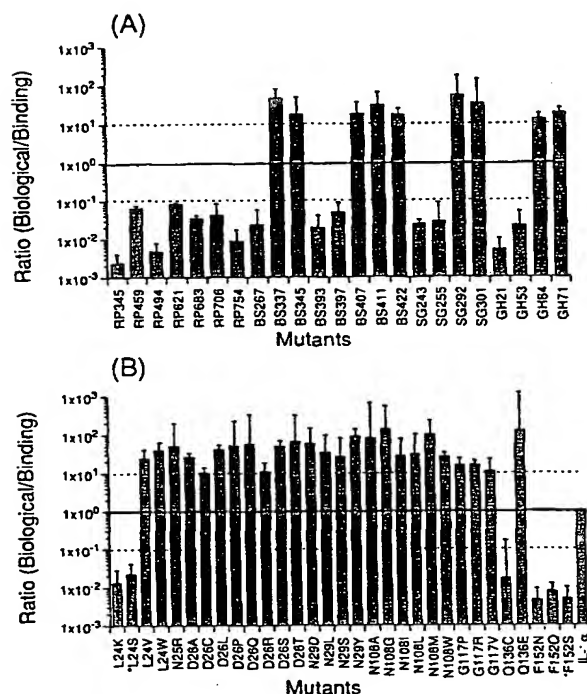


FIG. 3. Mutants with altered activities. The activity ratios were normalized to the internal wild-type IL-1 α controls and the mean from at least three screens for mutants with normalized ratios 10-fold greater or less than IL-1 α are shown. Panel A, proteins generated by saturation mutagenesis. Panel B, proteins generated by site-directed random mutagenesis at single amino acid residues. The single letter amino acid code is used to describe the amino acid in wild-type IL-1 α , the number of the residue, and the amino acid of the mutant. An asterisk indicates single amino acid changes that were also isolated from the saturation mutagenesis screens.

a cassette inserted. The ligation mixture was transformed into GM1[pRK248]. The transformants were screened for insertion of the mutagenic cassette by colony hybridization (Poindexter *et al.*, 1991). Double-stranded sequencing of the vectors with inserts was performed using the dideoxy method (Sanger *et al.*, 1977).

Protein Expression and Analysis—Mutant proteins were produced by using a pH induction protocol (Poindexter and Gayle, 1991). Cells containing recombinant plasmids were inoculated into 24-well plates containing 1 ml of Superbroth (Ausubel *et al.*, 1988), supplemented with M9 minimal salts and 1% glucose. Following overnight growth at 30 °C, the pH of the medium was shifted to 9 by the addition of 5 M NaOH. The cells were grown at 30 °C at pH 9 for 18 h before the cells were pelleted. Cells (40 μ l) were spun down in a 96-well plate at 3,000 rpm for 15 min. The cells were resuspended in an equal volume of lysis buffer (125 mM Tris, pH 8, 2% SDS) and then 80 μ l of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂HPO₄, pH 7.4) was added. The samples were then used for biological or binding assays. Binding activity was determined by the capacity of *Escherichia coli* cell lysates to inhibit the binding of radiolabeled IL-1 α to EL4 cells (Mosley *et al.*, 1987b). Biological activity was examined using an EL4 conversion assay (Mosley *et al.*, 1987a). *E. coli* lysates containing wild-type IL-1 α were included in every assay as controls. The data were analyzed by a nonlinear least squares fitting routine, and activities were quantified using a standard curve, derived from purified IL-1 α , for each biological assay and each binding assay.

Modeling IL-1 α Structure—The three-dimensional model of IL-1 α used in this study is an all atom protein model built using the C α coordinates generated from the stereo diagrams of the crystal structure (Graves *et al.*, 1990). The X and Y coordinates for all of the C α atoms were measured in the user unit using one of the two figures in the stereo diagram. The known standard distance of 3.8 Å, expected between the successive C α atoms, was used to scale and assign the Z coordinates for all of the C α atoms. An all atom model of 159 residues

was constructed in FRODO (Jones, 1985) from these 151 C α coordinates (Leu-7 to Asn-157). The structure was refined in a modified version of Biograf (Peebles and Goldstein, 1989).

Distances between the successive C α atoms were computed in the X-Y projection by measuring the X and Y coordinates from one of the two stereo diagrams. A value of +1, -1, or 0 was assigned to the Z coordinates of every C α atom if it was above, below, or in the same depth as the previous C α atom in the stereo view. The scale factor required to change the user unit to Å unit was computed by equating the computed projection distance between two successive C α atoms, with a sign of 0, to that of the standard distance of 3.8 Å. The X and Y coordinates of all of the C α atoms were multiplied with this scale factor, and the projection distance between the successive C α coordinates was computed. All of the projection distances measured this way will be either less than or equal to 3.8 Å because of the Z flattening. Appropriate Z coordinates were then assigned to each C α atom such that the computed distance between successive C α measured 3.8 Å. The computed Z coordinates were multiplied with the sign assigned to them. The cumulative error in the computation of the Z coordinates was corrected by adjusting only the Z coordinates using a wire model. The accuracy of the computed coordinate was tested by superimposing the resulting C α trace on to the structurally conserved regions of IL-1 β structure. The root mean square value for the structurally conserved region is 0.75 Å.

RESULTS

Alteration of the IL-1 α Gene by Saturation Mutagenesis—The synthetic IL-1 α gene has unique restriction enzyme sites approximately every 60 base pairs, dividing the gene into eight different regions (Fig. 1). The mutants from each region of the molecule are described by the restriction enzymes found at each end of the region. Thus the eight regions are CR (*Clai-EcoRI*), RP (*EcoRI-PstI*), PS (*PstI-SstI*), SU (*SstI-PvuII*), UB (*PvuII-BamHI*), BS (*BamHI-SpeI*), SG (*SpeI-BglI*), and GH (*BglI-HindIII*). Saturation mutagenesis using cassettes for each region was performed throughout the entire molecule, relying on a technique that results in very low levels of wild-type sequences and roughly equal probabilities of one to five nucleotide changes in any one region (Poindexter *et al.*, 1991). The use of intermediate plasmids in the constructions, along with colony hybridizations (see "Materials and Methods"), greatly improved the yield of recombinant vectors, allowing insert frequencies over 90% to be achieved. More than 3,500 mutants were generated by this approach, encompassing the eight regions of the IL-1 α gene (Fig. 1).

In one region (RP), 30 mutants were sequenced to determine accurately the mutation frequencies (Poindexter *et al.*, 1991). Those 30 mutants averaged 2.5 amino acid changes each, and every amino acid that could be altered in this region was found to be changed at least once. Several amino acids had three or four different substitutions. Sequencing mutants in each of the other seven regions did not reveal any deviation from the expected mutation frequency. More than 110 mutants were sequenced, and approximately 70% of the amino acid residues in IL-1 α were altered at least once. At this rate of mutagenesis, screening approximately 520 mutants, or roughly 65 mutants from each of the eight regions, should result in a 99% chance that all of the amino acids in IL-1 α which could be changed were altered at least once (for a discussion of calculating these probabilities, see Hutchison *et al.*, 1986). Thus, examination of 3,500 mutants should sample multiple mutations at every possible amino acid.

Screening of Mutant IL-1 α Proteins—Determining both the biological activity and the ability to inhibit the binding of wild-type IL-1 α to the type I IL-1 receptor for each mutant allowed molecules to be identified which affected these two functions differentially. The ratio of biological activity to binding ability was examined. This ratio represents an intrinsic specific activity of the molecule and therefore should be independent of protein concentration. To validate this ap-

Identification of Regions in IL-1 α Important for Activity

FIG. 4. Sequence of mutants that alter activity. The amino acid sequence of the relevant cassettes is shown with the amino acid changes for each mutant underneath. The single letter amino acid code is used.

RP Mutants	I	L	N	D	A	L	N	Q	S	I	I	R	A	N	D	Q	Y	L	T
RP345				D				M										D	
RP459					E			W											
RP494												M	P	S				N	
RP621					E						T								
RP683									L		S								
RP706					E							M							A
RP754/761				S															
BS Mutants	S	E	T	N	L	L	F	F	W	E	T	H	G	T	K	N	Y	F	T
BS267				T										D				F	
BS337					R								Q	V					
BS345				Y							K								P
BS393					P		S									K	F		
BS397								D				D		I					
BS407		A		I				Y										H	
BS411				K						D								D	
BS422							L			V		D							
SG Mutants	S	V	A	H	P	N	L	F	I	A	T	K	Q	D	Y	W	V	C	L
SG243					Y									L					
SG255											S							L	
SG292												T			H				
SG301					T							N							S
GH Mutants	A	G	G	P	P	S	I	T	D	F	Q	I	L	E	N	Q	A		
GH21											S								
GH53					L						V	H							
GH64						W	L					K							K
GH71						S	S												

proach, the biological activity and binding activity of 96 identical samples of an *E. coli* lysate containing wild-type IL-1 α were determined (Fig. 2A). Plotting the biological activity against the binding activity resulted in a cluster of points, with the greatest error being in the biological activity, presumably because of the greater inherent variation in this assay than in the binding assay. Screening *E. coli* lysates from 96 different inductions, each producing wild-type IL-1 α , resulted in the greater scatter with a tendency for the points to cluster along a line whose slope equaled the activity ratio of wild-type IL-1 α (Fig. 2B). Altering the concentration of wild-type IL-1 α only moves the ratio along this line. Dilutions were performed to verify that the activity seen was linear with respect to IL-1 α concentration (data not shown). The assays were able to distinguish levels of biological and binding activity over a range greater than 1,000-fold.

Mutants with a wild-type activity ratio should fall along the same line as wild-type IL-1 α controls included in each assay. Proteins with increased biological activity in comparison to the amount of binding activity seen should fall above this line, whereas mutant proteins with decreased amounts of biological activity compared with the binding activity seen should fall below this line. For screening purposes, mutants were determined to have altered activity ratios if there was at least a 10-fold increase or decrease in the ratio relative to wild-type IL-1 α for at least three independent sets of assays. In addition, mutants whose *E. coli* lysates lacked activity in either assay were rescreened to verify the lack of activity.

Approximately 1,700 clones were examined from the CR, PS, SU, and UB regions, encompassing more than one-half of the molecule. None of the mutants that demonstrated

activity displayed any significant deviation from wild-type levels. These regions have an average of 2.2 amino acid changes per mutant (data not shown), which is not significantly different from the expected mutation rate. Fig. 2C displays a set of typical data for 90 mutants from the PS region of the molecule. The majority of these proteins have activity ratios similar to wild-type IL-1 α . The other three regions give similar profiles.

Although most of the 1,800 mutants examined from the other four regions (RP, BS, SG, and GH) had activity ratios within 10-fold of wild-type IL-1 α , each region included several mutants that had a ratio that deviated from the wild-type IL-1 α ratio by more than 10-fold. An analysis of 90 typical mutants from the RP region is shown in Fig. 2D. There are several mutants in this group which have significantly different activity ratios from wild-type IL-1 α . Although several of these mutants failed to maintain an altered activity ratio upon subsequent assays, many continued to have altered activity ratios.

Mutants from saturation mutagenesis with activity ratios 10-fold higher or 10-fold lower than wild-type are shown in Fig. 3A. IL-1 α showed itself to be extremely resilient to change. Only 24 unique DNA sequences, out of more than 3,500 examined, produced protein that displayed a significant difference in activity from wild-type (Fig. 3A). This represents less than 0.7% of the mutants examined.

Of the 24 different mutants with altered ratios, 23 had unique amino acid sequences (Fig. 4). RP754 and RP761, although having the same amino acid sequence, have different DNA sequences. This demonstrates the power of this approach since it was possible to identify two independent clones

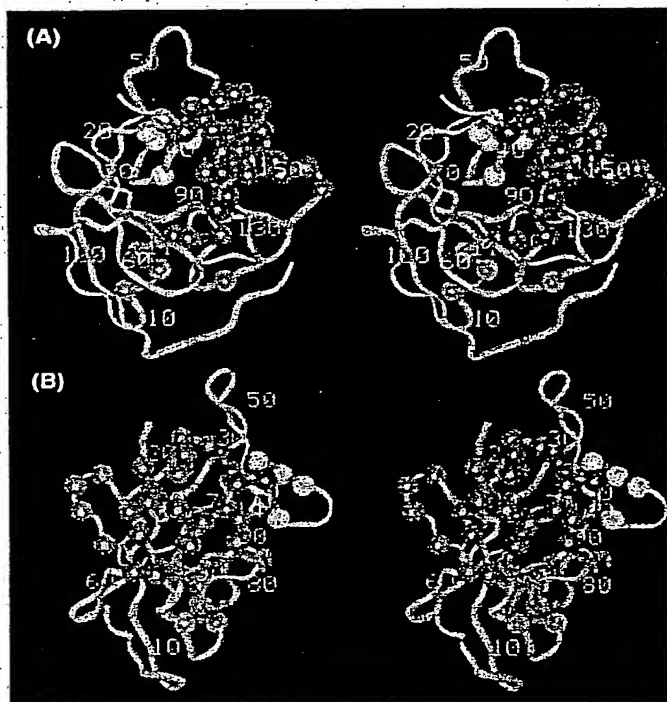


FIG. 5. Three-dimensional model of IL-1 α . Stereoview showing position of residues that affect the activity of the molecule. Panel A, the plane of the figure is roughly perpendicular to the axis of the 12-stranded barrel. Panel B, rotated 90° from view in panel A. The α -carbon backbone is shown as a ribbon, with every 10th residue numbered. The green spheres illustrate the major region identified. The red and yellow spheres indicate the smaller regions made up of residues Glu-106, Asn-108, Leu-109, His-127, and Ile-33, Arg-34, Ala-35, Tyr-39, Thr-41, and respectively.

that had the same phenotype and the same amino acid changes out of a large pool of recombinants. The 23 distinct amino acid sequences contained mutations at a total of 38 amino acid residues. This corresponds to approximately 24% of the molecule.

Random Site-directed Mutagenesis—Because saturation mutagenesis often results in conservative amino acid changes, random mutagenesis was performed at several sites in the molecule. During the synthesis of a particular mutagenic cassette, a random sequence was placed at the codon of interest, allowing all possible amino acids to be produced at this position. Site-directed mutagenesis was performed at residues in the four regions identified by saturation mutagenesis. Leu-24 and Phe-152 were chosen because single amino acid changes introduced at these positions had already been found to alter the activity ratio (i.e. mutants RP754 and GH21). Asp-26, Leu-28, Asn-108, Gly-117, and Tyr-121 were examined because more than one mutant had been found which altered these residues. Asn-25 and Gln-136 were studied because they appeared to be conserved hydrophilic residues that were exposed to solvent, according to the crystallographic data (Graves *et al.*, 1990). Asn-29 was also mutated because it was one of the few conserved residues in the RP region of the molecule which was not identified using saturation mutagenesis. Up to 100 mutants at each position were examined. Screening proteins generated by random mutagenesis identified several further single amino acid changes that affected the activity ratios of the molecule (Fig. 3B). Although none of the changes at Leu-28 or Tyr-121 appeared to affect the ratio of biological activity to binding activity, substitutions at

each of the other 8 amino acids produced at least one mutant that altered the activity ratio.

Spatial Location of Mutations—The crystal structures of IL-1 α (Graves *et al.*, 1990) and IL-1 β (Gilliland *et al.*, 1987; Priestle *et al.*, 1990) have been determined to 2.7 and 2.0 Å, respectively. Using a novel approach, the three-dimensional coordinates were determined from the published structure. The model of IL-1 α superimposes on the similar trace from IL-1 β with a root mean square difference of 0.75 Å. Residues that were determined by saturation mutagenesis and by random mutagenesis to affect activity ratios are displayed on the model for IL-1 α (Fig. 5). Interestingly, these amino acids cluster in three regions. The majority are found along one side of the molecule, encompassing an area of approximately 600 Å².

DISCUSSION

More than 3,500 mutants were generated throughout IL-1 α by saturation mutagenesis. The rate of mutagenesis was more than sufficient to produce several amino acid changes at every possible residue in these 3,500 mutants. The biological activity and the ability to inhibit the binding of IL-1 α were measured for every mutant. The ratio of biological activity to binding activity gives a measure of the specific activity of each mutant. Antagonists will have low ratios, whereas mutants with high ratios demonstrate enhanced agonist activity.

Most of the molecule could be mutated with little effect on either activity. Combining the data from saturation mutagenesis and site-directed mutagenesis, alterations at only 39 positions resulted in proteins with modified activity ratios. Although mutations at these residues resulted in proteins with activity ratios up to 1,000-fold less than wild-type, it would appear that only a limited number of residues are critically required for activity. Most of the other 139 residues, or 75% of the molecule, may not contribute significantly to the biological activity of the molecule. As much as 68% of IL-1 α may have little informational content, allowing a wide variety of amino acids to be substituted with little effect on activity (Zurawski, 1991). This is consistent with the observations reported in this paper.

Fig. 5 shows the spatial locations of the 39 residues changed in mutants with altered activity ratios. Almost all of the amino acid changes were found in β -strands, not in loops. With the exception of amino acids in strands 1 and 12, most of these amino acid residues have not been identified previously as important for activity. More than 75% of the identified amino acid residues are located along one face of the molecule. A substantial number of the mutated residues are located in β -strands 1, 2, 8, 9, 11, and 12. Several residues that may be involved in determining the activity of IL-1 α , such as Asp-26, Lys-119, Gln-136, Ile-149, and Asp-151, appear in spatially similar positions in IL-1 β .

In addition to this one major region there appear to be two smaller areas: one that includes Glu-106, Asn-108, Leu-109, and His-127, and another involving residues Ile-33, Arg-34, Ala-35, Tyr-39, and Thr-41. The former three amino acids form a small hydrophilic patch at the bottom and slightly behind the large region of mutated residues, whereas the latter amino acids form an exposed patch off to one side, separated from the main region by strands 3 and 4. The identification of three regions important for activity is intriguing. Since the IL-1 receptor is composed of three IgG-like domains, it has been postulated that each of the three domains interacts with a region on IL-1 (Cloré *et al.*, 1991). Deletion of any of these three domains greatly reduces the binding of ligand (Dower and Sims, 1990).

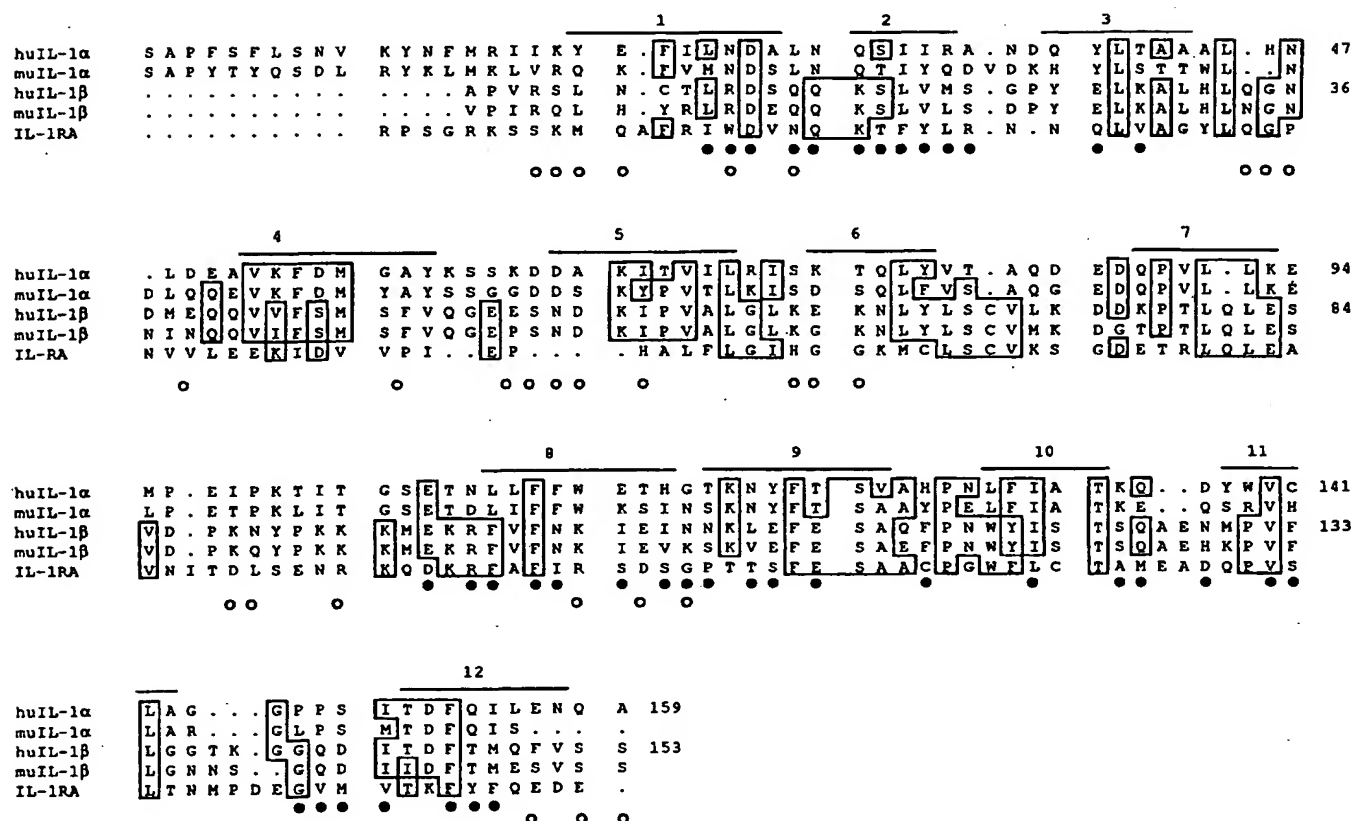


FIG. 6. Alignment of human and murine IL-1 α , human and murine IL-1 β , and IL-1 receptor antagonist. Residues that are conserved in at least three of the sequences are boxed. The β -strands for IL-1 α are shown above the sequence. The numbering for human IL-1 α and IL-1 β is displayed on the right. Closed circles indicate amino acid residues that were changed in IL-1 α mutants with altered phenotypes. Open circles indicate residues in human IL-1 β which have been postulated to interact with the type I IL-1 receptor.

Using site-directed mutagenesis, single amino acid changes that decrease or increase the ratio of biological activity to binding ability were found at several positions in the molecule. At most of these locations only the substitution of specific amino acids had any impact on activity. Screening up to 100 isolates revealed only a small number of specific amino acid substitutions at Leu-24, Asn-25, Asn-29, Gly-117, Gln-136, and Phe-152 which altered the activity ratio. Substitutions at Asp-26 and Asn-108 were exceptions. The presence of any of 10 amino acids at Asp-26 altered the activity ratio. Changes at Asp-26 which reduce the amount of biological activity have been reported (Yamayoshi *et al.*, 1990). This may indicate that an Asp residue at position 26 is required for wild-type activity. Several hydrophilic substitutions at Asn-108 did not appear to affect biological activity (Zurawski, 1991). However, placing hydrophobic residues at this position did affect the ratio, with a relative increase in the biological activity.

Few other reports of IL-1 α mutagenesis have distinguished between changes that affect biological activity and those that also alter the interactions of the protein with the receptor. Mutations at Asp-151 differentially affect biological activity and binding ability (Yamayoshi *et al.*, 1990). Examination of a large group of IL-1 α mutants has previously revealed the importance of Asp-26 and Asp-151, but only the effects on biological activity were studied (Kawashima *et al.*, 1992). Random combinatorial mutagenesis has identified amino acids in which substitutions have no effect on biological activity (Yanofsky and Zurawski, 1990). The lack of muta-

tions at certain residues was used to infer the importance of these residues for biological activity. There was, however, no demonstration of the ability of these mutants to interact with the IL-1 receptor. Since only biological activity was examined, it is difficult to determine whether the constraints on amino acid residues were functional in nature or whether there were structural constraints as well.

Sequence alignments of human and mouse IL-1 α and IL-1 β are shown in Fig. 6. These molecules bind to the type I IL-1 receptor and are active in the EL4 conversion assay. Inclusion of IL-1ra, a molecule that also binds to IL-1 receptor but produces no biological activity, in the alignment does not immediately reveal any obvious region that is responsible for the uncoupling of biological activity and the ability to bind receptor. The 39 amino acids identified in this report are also shown. Although many of these amino acids are found toward the COOH terminus of the molecule (which is the most highly conserved region of the molecule), there does not appear to be much selection for conserved residues. In fact, regions in which few amino acids are conserved, such as in strand 2 or strand 8, have several residues that appear to be important for activity. Although there are no data demonstrating that IL-1 α and IL-1 β interact with the type I IL-1 receptor in the same fashion, residues of IL-1 β which have been shown to be important for biological activity are found in positions homologous to some of the residues identified for IL-1 α (Gehrke *et al.*, 1990; Ju *et al.*, 1991).

Interestingly, residues of IL-1 β which have been shown to

influence binding to the type I IL-1 receptor or have been proposed to interact with the receptor (Clare *et al.*, 1991; Grenfell *et al.*, 1991; Labriola-Tompkins *et al.*, 1991; Veerapandian *et al.*, 1992) are generally located outside the regions of IL-1 α shown in Fig. 5. Out of 45 amino acids postulated to be involved in binding of IL-1 β only 9 overlap with residues identified in this study (Fig. 6). IL-1 α and IL-1 β either have different regions interacting with the receptor, or there is a large region in IL-1 α and IL-1 β which is required for biological activity but not for high affinity interactions with the receptor.

A superfamily of molecules with protein folding similar to IL-1 has been proposed (Graves *et al.*, 1990; Murzin *et al.*, 1992). This superfamily includes certain proteinase inhibitors and heparin-binding growth factors, such as fibroblast growth factor. Two forms of fibroblast growth factor have been shown to fold in a very similar fashion to IL-1 α and IL-1 β (Ago *et al.*, 1991; Eriksson *et al.*, 1991; Zhang *et al.*, 1991; Zhu *et al.*, 1991), displaying 12 β -strands with a pseudo 3-fold symmetry. Several regions of the fibroblast growth factor molecule have been identified which are important for activity. These areas occupy spatially similar regions of the fibroblast growth factor three-dimensional structure as the amino acids of IL-1 α identified by saturation mutagenesis. The modes of interaction between members of the IL-1 superfamily and their respective receptors may involve similar regions of the folded protein.

The manner in which IL-1 generates a biological response is complicated. There are two different ligands, an IL-1 antagonist and two forms of IL-1 receptor (Dower *et al.*, 1990). There is evidence for multiple pathways of signal transduction (for review see Sims *et al.*, 1993). The effect of IL-1 on a particular cell type may depend on the receptor found on the cell and which signaling pathway is being used. Analysis of the effect of these mutations on the different biological responses may be helpful in further elucidation of IL-1 signal transduction.

REFERENCES

- Ago, H., Kitagawa, Y., Fujishima, A., Matsuura, Y., and Katsube, Y. (1991) *J. Biochem.* 110, 360-363.
- Arend, W. P., Welgus, H. G., Thompson, R. C., and Eisenberg, S. P. (1990) *J. Clin. Invest.* 85, 1694-1697.
- Auron, P. E., Warner, S. J., Webb, A. C., Cannon, J. G., Bernheim, H. A., McAdam, K. J., Rosenwasser, L. J., LoPrete, G., Mucci, S. F., and Dinarello, C. A. (1987) *J. Immunol.* 138, 1447-1456.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. J., Smith, J. A., and Struhl, K. (1988) *Current Protocols in Molecular Biology*, John Wiley and Sons, New York.
- Carter, D. B., Deibel, M. J., Dunn, C. J., Tomich, C. S., Laborde, A. L., Slightom, J. L., Berger, A. E., Bienkowski, M. J., Sun, F. F., McEwan, R. N., Harris, P. K. W., Yem, A. Y., Waszak, G. A., Chosay, J. G., Sieu, L. C., Hardee, M. M., Zurcher-Neely, H. A., Reardon, I. M., Heinrichson, R. L., Truesdell, S. E., Shelly, J. A., Eessalu, T. E., Taylor, B. M., and Tracey, D. E. (1990) *Nature* 344, 633-638.
- Clare, G. M., Wingfield, P. T., and Gronenborn, A. M. (1991) *Biochemistry* 30, 2315-2323.
- Craig, S., Pain, R. H., Schmeissner, U., Virden, R., and Wingfield, P. T. (1989) *Int. J. Pept. Protein Res.* 33, 256-262.
- Dower, S. K., and Sims, J. E. (1990) in *Cellular and Molecular Mechanisms of Inflammation* (Cochrane, C. G., and Gimbrome, J. M. A., eds) pp. 137-172. Academic Press, New York.
- Dower, S. K., Kronheim, S. R., Hopp, T. P., Cantrell, M., Deeley, M., Gillis, S., Henney, C. S., and Urdal, D. L. (1986) *Nature* 324, 266-268.
- Dower, S. K., Bomzstyk, K., and Sims, J. E. (1990) *Prog. Clin. Biol. Res.* 349, 241-249.
- Durum, S. K., Schmidt, J. A., and Oppenheim, J. J. (1985) *Annu. Rev. Immunol.* 3, 263-287.
- Eisenberg, S. P., Evans, R. J., Arend, W. P., Verderber, E., Brewer, M. T., Hannum, C. H., and Thompson, R. C. (1990) *Nature* 343, 341-346.
- Eriksson, A. E., Cousins, L. S., Weaver, L. H., and Matthews, B. W. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 3441-3445.
- Gehrke, L., Jobling, S. A., Paik, L. S., McDonald, B., Rosenwasser, L. J., and Auron, P. E. (1990) *J. Biol. Chem.* 265, 5922-5925.
- Gilliland, G. L., Winborne, E. L., Masui, Y., and Hirai, Y. (1987) *J. Biol. Chem.* 262, 12323-12324.
- Graves, B. J., Hatada, M. H., Hendrickson, W. A., Miller, J. K., Madison, V. S., and Satow, Y. (1990) *Biochemistry* 29, 2679-2684.
- Grenfell, S., Smithers, N., Witham, S., Shaw, A., Graber, P., and Solari, R. (1991) *Biochem. J.* 280, 111-116.
- Gronenborn, A. M., Wingfield, P. T., McDonald, H. R., Schmeissner, U., and Clare, G. M. (1988) *FEBS Lett.* 231, 135-138.
- Hannum, C. H., Wilcox, C. J., Arend, W. P., Joslin, F. G., Dripps, D. J., Heidmal, P. L., Armes, L. G., Sommer, A., Eisenberg, S. P., and Thompson, R. C. (1990) *Nature* 343, 336-340.
- Hutchison, C. A. I., Nordeen, S. K., Vogt, K., and Edgell, M. H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 710-714.
- Jones, T. A. (1985) *Methods Enzymol.* 115, 157-171.
- Ju, G., Labriola-Tompkins, E., Campen, C. A., Benjamin, W. R., Karas, J., Plocinski, J., Biondi, D., Kaffka, K. L., Kilian, P. L., Eisenberg, S. P., and Evans, R. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 2658-2662.
- Kamogashira, T., Masui, Y., Ohmoto, Y., Hirato, T., Nagamura, K., Mizuno, K., Hong, Y. M., Kikumoto, Y., Nakai, S., and Hirai, Y. (1988a) *Biochem. Biophys. Res. Commun.* 150, 1106-1114.
- Kamogashira, T., Sakaguchi, M., Ohmoto, Y., Mizuno, K., Shimizu, R., Nagamura, K., Nakai, S., Masui, Y., and Hirai, Y. (1988b) *J. Biochem. (Tokyo)* 104, 837-840.
- Kawashima, H., Yamagishi, J.-i., Yamayoshi, M., Ohue, M., Fukui, T., Kotani, H., and Yamada, M. (1992) *Protein Eng.* 5, 171-176.
- Labriola-Tompkins, E., Chandran, C., Kaffka, K. L., Biondi, D., Graves, B. J., Hatada, M., Madison, V. S., Karas, J., Kilian, P. L., and Ju, G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 11182-11186.
- March, C. J., Mosley, B., Larsen, A., Cerretti, D. P., Braedt, G., Price, V., Gillis, S., Henney, C. S., Kronheim, S. R., Grabstein, K., Conlon, P. J., Hopp, T. P., and Cosman, D. (1985) *Nature* 315, 641-647.
- McMahan, C. J., Slack, J. L., Mosley, B., Cosman, D., Lupton, S. D., Brunton, L. L., Grubin, C. E., Wignall, J. M., Jenkins, N. A., Brannan, C. I., Copeland, N. G., Huebner, K., Croce, C. M., Cannizzaro, L. A., Benjamin, D., Dower, S. K., Spriggs, M. K., and Sims, J. E. (1991) *EMBO J.* 10, 2821-2832.
- Mosley, B., Dower, S. K., Gillis, S., and Cosman, D. (1987a) *Proc. Natl. Acad. Sci. U. S. A.* 84, 4572-4576.
- Mosley, B., Urdal, D. L., Prickett, K. S., Larsen, A., Cosman, D., Conlon, P. J., Gillis, S., and Dower, S. K. (1987b) *J. Biol. Chem.* 262, 2941-2944.
- Murzin, A. G., Lesk, A. M., and Chothia, C. (1992) *J. Mol. Biol.* 223, 531-543.
- Peeples, W. J., and Goldstein, P. (1989) *Cytobios* 58, 109-123.
- Poindexter, K., and Gayle, R. B., III. (1991) *Gene (Amst.)* 9, 125-130.
- Poindexter, K., Jerzy, R., and Gayle, R. B., III. (1991) *Nucleic Acids Res.* 19, 1899-1904.
- Priestle, J. P., Schar, H. P., and Grutter, M. G. (1990) *Prog. Clin. Biol. Res.* 349, 297-307.
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463-5467.
- Sims, J. E., Bird, T. A., Giri, J. G., and Dower, S. K. (1993) *IL-1 Signal Transduction: Signal Transduction through Growth Factor Receptors* (Kitagawa, Y., ed), Jai Press, Greenwich, in press.
- Veerapandian, B., Gilliland, G. L., Raag, R., Svensson, A. L., Masui, Y., Hirai, Y., and Poulos, T. L. (1992) *Protein Struct. Funct. Genet.* 12, 10-23.
- Yamayoshi, M., Ohue, M., Kawashima, H., Kotani, H., Iida, M., Kawata, S., and Yamada, M. (1990) *Lymphokine Res.* 9, 405-413.
- Yanofsky, S. D., and Zurawski, G. (1990) *J. Biol. Chem.* 265, 13000-13006.
- Zhang, J. D., Cousins, L. S., Barr, P. J., and Sprang, S. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 3446-3450.
- Zhu, X., Komiya, H., Chirino, A., Faham, S., Fox, G. M., Arakawa, T., Hsu, B. T., and Rees, D. C. (1991) *Science* 251, 90-93.
- Zurawski, G. (1991) *Trends Biotechnol.* 9, 250-257.

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